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Exhibit B

A single-injection protein kinase A-directed antisense treatment to inhibit tumour growth

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Expression of the RI_α subunit of cAMP-dependent protein kinase type I is enhanced in human cancer cell lines, in primary tumours, in cells after transformation and in cells upon stimulation of growth. We have investigated the effect of sequence-specific inhibition of RI_α gene expression on *in vivo* tumour growth. We report that single injection RI_α antisense treatment results in a reduction in RI_α expression and inhibition of tumour growth. Tumour cells behaved like untransformed cells by making less protein kinase type I. The RI_α antisense, which produces a biochemical imprint for growth control, requires infrequent dosing to halt neoplastic growth *in vivo*.

Standard cytotoxic chemotherapy for cancer is usually accompanied by systemic toxicity. The ratio of the toxic dose to the therapeutic dose is relatively low, reflecting the large number of cellular targets affected by the chemotherapeutic agent. In principle, because of the specificity of Watson-Crick base pairing, an antisense oligonucleotide targeted at a gene involved in the neoplastic cell growth should interfere only with that gene's expression, resulting in arrest of cancer cell growth.

Enhanced expression of the RI_α subunit of cAMP-dependent protein kinase (PKA)[1] has been shown in human cancer cell lines and in primary tumours, as compared with normal counterparts, in cells after transformation with the Ki-*ras* oncogene or transforming growth factor-α, and upon stimulation of cell growth with granulocyte-macrophage colony-stimulating factor (GM–CSF) or phorbol esters[2,3]. Conversely, a decrease in the expression of RI_α correlates with growth inhibition induced by site-selective cAMP analogues in a broad spectrum of human cancer cell lines[3].

There are two types of PKA, type I (PKA-I) and type II (PKA-II), which share a common C subunit but contain distinct R subunits, RI and RII, respectively[4]. Through biochemical studies and gene cloning, four isoforms of the R subunits, RI_α, RI_β, RII_α and RII_β, have been identified[5,6]. Three distinct C subunits, C_α (ref. 7), C_β (refs 8, 9) and C_γ (ref. 10) have also been identified; however, preferential coexpression of one of these C subunits with any of the R subunits has not been found[9,10]. The R isoforms differ in tissue distribution[11,12] and in biochemical properties[4,13]. The two general isoforms of the R subunit also differ in their subcellular localization. RI is found throughout the cytoplasm, whereas RII localizes to nuclei, nucleoli, Golgi apparatus and the microtubule-organizing center[2,14,15]. The expression of RI/PKA-I and RII/PKA-II has an inverse relationship during ontogenic development and cell differentiation[2,3]. However, the significance of the presence of these two isoforms of PKA in the biological functions of cAMP has not been determined.

We hypothesize that the RI_α is an ontogenic growth-inducing protein and that its constitutive expression disrupts normal ontogenic processes, resulting in a pathogenic outgrowth, such as malignancy. The two general classes of PKA R subunits, RI and RII, have conserved amino acid sequences at the carboxyl terminus but differ significantly at the amino terminus[16]. An RI_α antisense phosphorothioate oligodeoxynucleotide corresponding to the N-terminal 8–13 codons of RI_α was constructed (RI_α antisense). We then investigated the effect of sequence-specific inhibition of RI_α gene expression on *in vivo* tumour growth.

Results

Inhibition of tumour growth

A single subcutaneous (s.c.) injection into nude mice bearing LS-174T human colon carcinoma with RI_α antisense resulted in almost complete suppression of tumour growth for 7 days as assessed either by tumour volume (Fig. 1*a*) or by tumour weight (Fig. 1*b*). There was no apparent sign of toxicity as evaluated by hematocrit levels, body weight and food intake. Even after 14 days, tumour growth was significantly inhibited in the antisense-treated animals (Fig. 1*b*). In contrast, tumours in saline-treated animals showed continued growth (Fig. 1), and tumours in untreated or control antisense-treated animals grew at a rate similar to those in saline-treated animals. The results of tumour growth inhibition were confirmed with three additional RI_α antisense constructs (each of the 21-base polymers directed to codons 1–7, 14–20 and 94–100, respectively, of human RI_α), which previously have been shown to inhibit growth in a variety of human cancer cell lines[17,18].

We also did experiments using the RI_α 8–13 codon antisense two-base-pair-mismatched oligonucleotide (5′-GCG-CGC-CTC-CTC-GCT-GGC-3′) to substantiate further the specificity of the antisense oligonucleotide. The data revealed that antisense mismatched oligonucleotide was unable to inhibit tumour growth. (The average tumour weights (in milligrams) ± s.d. at 12 days post-treatment for mismatched oligonucleotide, scrambled oligonucleotide, and antisense oligonucleotide were 1533 ± 250 ($n = 5$), 1490 ± 305 ($n = 5$), and 439 ± 88 ($n = 5$), respectively.)

The LS-174T human colon carcinoma in this model behaved in a very aggressive manner. Within a week after tumour cell inoculation, a palpable tumour mass was formed, and thereafter tumours often killed host animals in 4–5 weeks. The results of these experiments provide clear evidence that the RI_α antisense phosphorothioate oligodeoxynucleotide has significant antitumour activity in an *in vivo* model system, where tumour growth was arrested for up to 14 days of observation after a single dose of antisense.

The human RI_α antisense (directed to 8–13 codons of human RI_α) also inhibited proliferation of the c-*ras*^{val12}-transformed mouse fibroblast cell line (K. Noguchi and Y.S.C.-C., unpublished results). The control antisense had no effect on the growth of the transformed fibroblasts. The human and mouse RI_α 8–13 codons contain one mismatched nucleotide at the 5′ end followed by a stretch of 10 nucleotides that are 100% homologous and contain four mismatches at the 3′ end[12,19]. Although the human RI_α 8–13 codons are not 100% homologous with the corresponding codons of the mouse RI_α, the antisense directed to this human sequence was apparently able to hybridize with the mouse RI_α mRNA, inducing growth inhibition. Thus, the lack of systemic toxicity in the RI_α antisense-treated animals was not due to the inability of the RI_α antisense to cross-react with the mouse RI_α gene.

Downregulation of RI_α

We examined whether the RI_α antisense could specifically decrease the amount of RI_α subunit in tumours (Fig. 2). At 0, 16 and 24 hours, 2, 3, 5 and 7 days after the single injection of RI_α antisense, the animals were killed, and tumours were analysed for the amount they contained of each of the R subunits of PKA. Tumour extracts were photoaffinity labelled with 8-N_3-[^{32}P]cAMP and then immunoprecipitated with the monospecific antibodies anti-RI_α, -RII_α and -RII_β (kindly provided by S.D. Park, Seoul National University, Seoul, South Korea), and the immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS–PAGE)[20]. The RI_α levels in tumours from the antisense-treated animals were markedly decreased within 24 hours and remained at low levels (10–20% of that in tumours from saline-treated animals) for up to 2–3 days (Fig. 2). Specific targeting of RI_α by the antisense is evident since RII_α levels remained unchanged (Fig. 2). At 5–7 days post-antisense treatment, the RI_α levels in tumours were elevated to levels similar to those in tumours from saline-injected animals (Fig. 2). At day 3 after antisense treatment, tumours that contained unreduced amounts of RI_α contained a new species of R, RII_β, along with a reduced amount of RII_α (Fig. 2). The increase in RII_β expression was also found in tumours that contained decreased levels of RI_α without reduction in RII_α content (data not shown). RII_β appeared 24 hours to 3 days post-antisense treatment but was not detected in control tumours (saline or control antisense-treated). These data show that the antisense-targeted suppression of RI_α brought about a compensatory increase in RII_β levels. Similar observations were made previously in cultured cancer cell lines upon treatment with RI_α antisense[17,18].

Immunoprecipitated RI_α and RII_β show a doublet, a major fast mobility band and a minor slow mobility one (Fig. 2). The doublets were specific bands because the addition of 1,000-fold excess cold cAMP added in the experiments using photoaffinity labelling with immunoprecipitation resulted in the disappearance of both bands. The slow mobility band of the RI_α doublet may be RI_β, which is highly homologous with RI_α but has a higher molecular weight than RI_α (ref. 12) and that the RII_β doublet may represent the autophosphorylated form of RII_β (ref. 21).

Elimination of PKA-I with induction of PKA-II_β

Upon antisense treatment, the level of RI_α, after its initial suppression for a few days, subsequently increased in tumours (Fig. 2). In cells, RI_α can exist either in its subunit form or in the form of the PKA-I holoenzyme. As the RI_α subunit can act as a

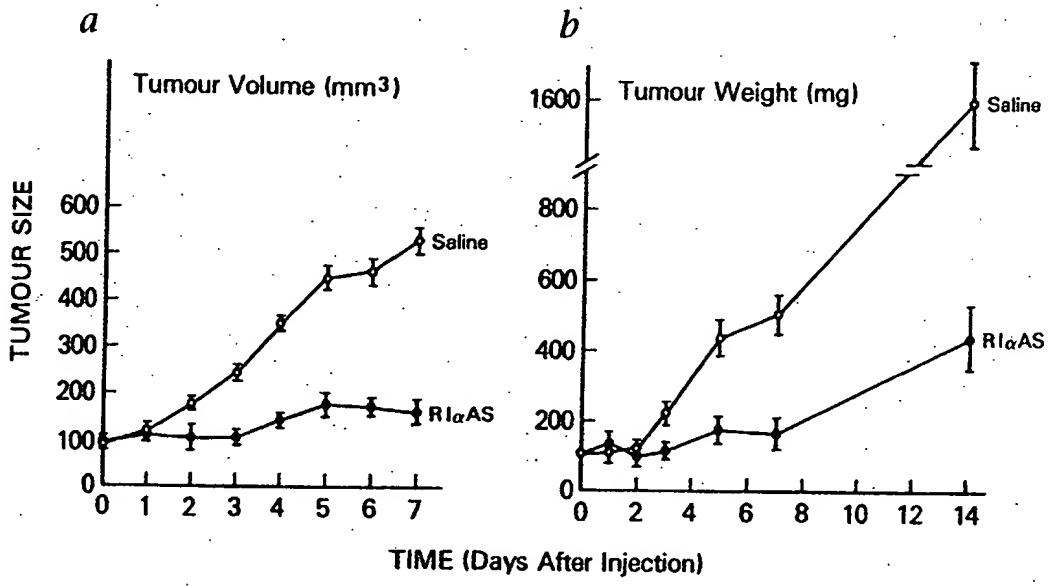

Fig. 1 Inhibition of *in vivo* tumour growth by a single dose of RI_α antisense. *a*, Tumour volume obtained from daily measurement. *b*, Tumour weight at the time mice were killed. Data represent means ± s.d. of 8–32 tumours in *a*; mean ± s.d. of 8 tumours in *b*. These data were obtained from four separate experiments where each experiment used 24 mice.

cAMP sink, RI_α in the holoenzyme complex may be of functional importance. We therefore examined whether RI_α antisense could influence the PKA isozyme distribution in tumours. We subjected tumour extracts to diethylaminoethyl (DEAE) ion-exchange chromatography, and fractions were assayed for PKA activity and cAMP-binding activity. Saline-treated control tumours showed two major peaks of PKA activity that were coincident with peaks of cAMP-binding activity. PKA-I eluted between 50 and 100 mM NaCl, whereas PKA-II eluted between 220 and 300 mM NaCl (Fig. 3, 24 hours post saline injection). In addition, there were two minor cAMP-binding peaks with no PKA activity eluted at 130 and 330 mM NaCl, respectively (Fig. 3). These were identified as RI_α and RII_α subunits, respectively, by photoaffinity labelling with 8-N_3-[^{32}P]cAMP (Fig. 4). Control tumours (24 hours to 7 days post saline injection or control antisense treatment) contained PKA-I and PKA-II in a 1:2 ratio (Fig. 3). The antisense treatment completely eliminated PKA-I, the RI_α-containing holoenzyme, and the RI_α subunit, from tumours within 24 hours (Fig. 3). This downregulation of PKA-I lasted for up to 5–7 days post-antisense treatment (Fig. 3) even when the RI_α levels increased until they reached the levels of control tumours (Figs. 2 and 3). This indicates that the RI_α that increased subsequently to its initial suppression after antisense treatment was mostly present in its subunit form rather than in its holoenzyme form, PKA-I.

Earlier reports demonstrated that brain and heart PKA-II eluted at slightly different salt concentrations from DEAE cellulose columns[4]. The brain contains high levels of RII_β, whereas the heart expresses the RII_α isoform. Heterogeneity of PKA-II has been reported and was attributed to either RII_2C trimer formation[24] or a RII_2C_2 tetramer with nonsaturating amounts of bound cAMP[25]. Otten *et al.*[26] identified three peaks of PKA-II in *ras*-transformed NIH3T3 (R3T3) cells overexpressing either mouse RII_α or rat RII_β; the first and third peaks of PKA-II were associated with RII_β and RII_α, respectively, and the second peak contained a mixture of RII_α and RII_β. Retroviral vector-mediated overexpression of the human RII_α or RII_β gene in LS-174T colon carcinoma cells has also shown three peaks of PKA-II; the first PKA-II is associated with RII_β, and the second and third peaks contain mainly RII_α[27].

Concomitant with the suppression of PKA-I, the antisense brought about changes in the PKA-II profile of tumours. The control tumours contained PKA-II with its main peak eluted at 260 mM NaCl (Fig. 3). Within 24 hours after antisense treatment, PKA-II with its peak eluted at 220 mM NaCl appeared prominently (Fig. 3). This altered PKA-II profile persisted in tumours for up to 2–3 days after antisense treatment, but by day 5, the PKA-II profile became similar to that of control tumours (Fig. 3). Photoaffinity labelling of the R subunits from tumours 24 hours after antisense treatment with 8-N_3-[^{32}P]cAMP showed the presence of RII_β only in fractions 33–37 (Figs 3 and 4). Immunoprecipitation of the R subunits confirmed their identity by photoaffinity labelling. Thus, PKA-II eluted at 220 mM NaCl (Fig. 3) was PKA-II_β. RII_β and PKA-II_β were detected in 50% of the tumours analysed 24 hours to 3 days after

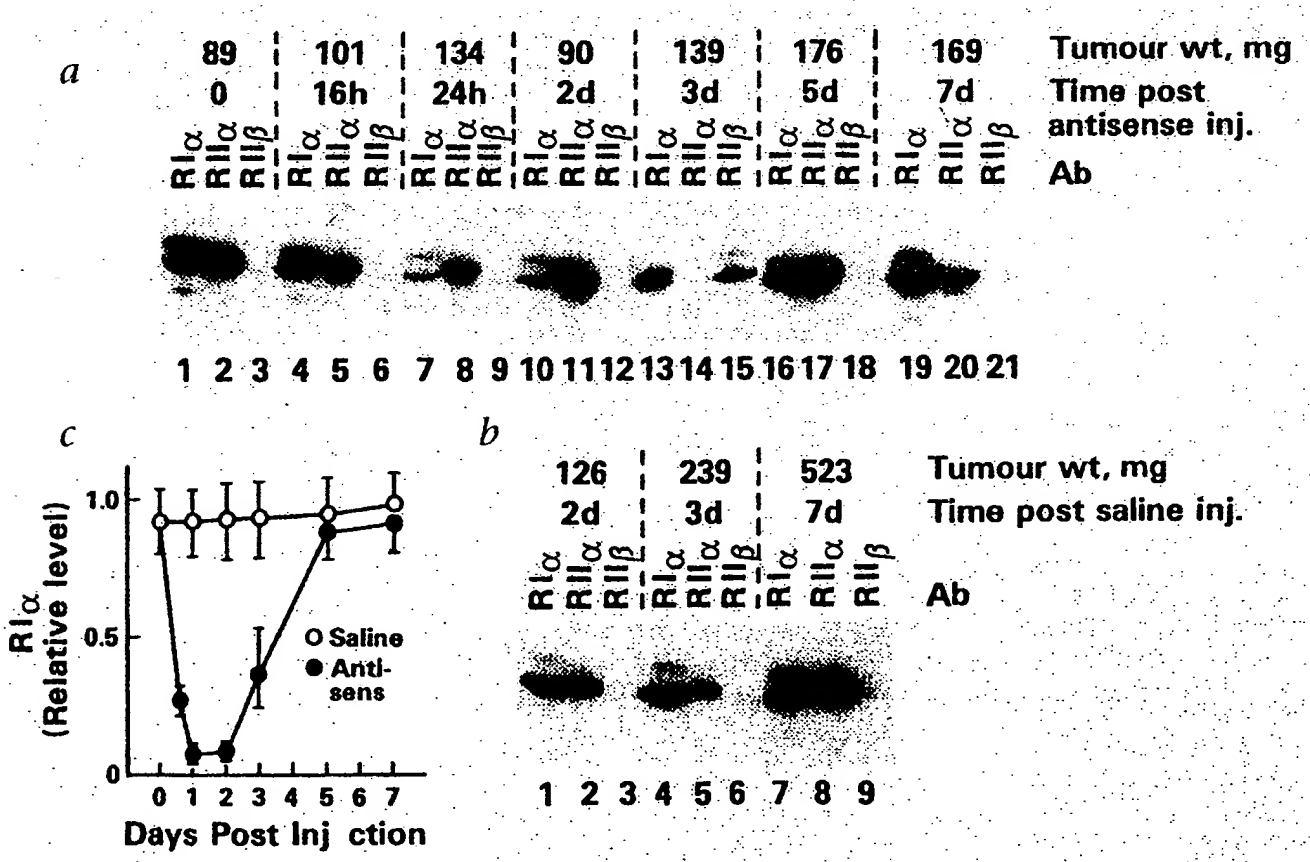

Fig. 2 Suppression of RI_α levels in tumours by a single dose of RI_α antisense. The R subunit levels in tumours were determined by photoaffinity labelling followed by immunoprecipitation. Gels in *a* and *b* represent one of four separate experiments that gave similar results. *c*, Quantification of RI_α levels by densitometric tracings of autoradiographs (*a* and *b*): the data represent relative average values ± s.d. of four experiments.

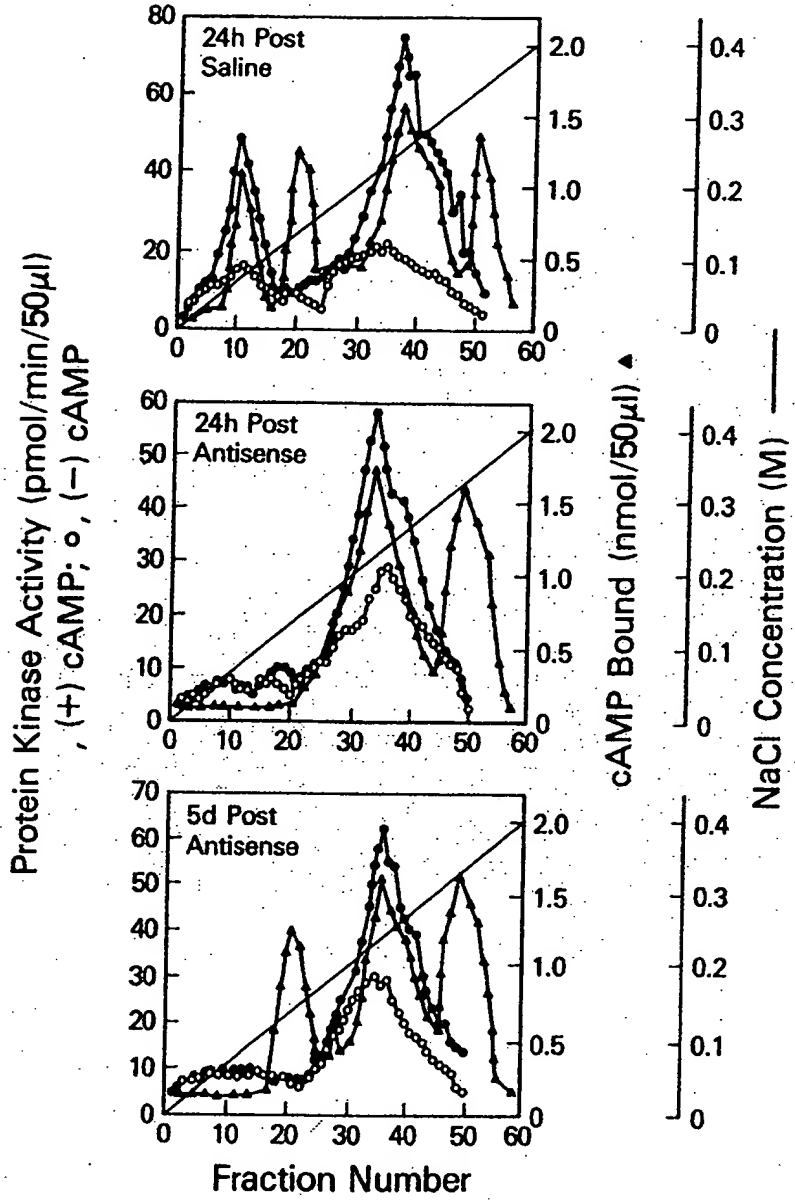

Fig. 3 PKA-I downregulation by a single dose of RI_α antisense: PKA activity in the absence (○) or presence (●) of 5 μM cAMP and cAMP-binding activity (▲) was measured in DEAE column eluents. Each chromatograph was repeated 3 or 4 times and yielded similar elution profiles.

antisense treatment but were not detected in control tumours (saline injection or control antisense treatment). These data suggest that the C subunits of PKA in tumours are in equilibrium between PKA-I and PKA-II and that deprivation of RI_α and PKA-I by the antisense led to an increase in PKA-II through the induction of PKA-II_β.

Increase of R subunit prote lysis

The RII_β expression in these tumours was accompanied by an increase in a low-molecular-weight R species, probably the proteolytically degraded R subunits[4,28] (Fig. 4). This apparent increase in R proteolysis was not an experimental artifact of tumour homogenization, because the homogenization buffer contained a cocktail of protease inhibitors. Furthermore, the low-molecular-weight R species were frequently detected in tumours 24 hours to 3 days after antisense treatment but were never observed in control tumours (saline or control antisense treated). Thus, the antisense treatment triggered RI_α downregulation as well as R subunit proteolysis, though the mechanism of these actions is not yet clear.

Discussion

We have demonstrated that a single injection of RI_α antisense resulted in an acute reduction in RI_α expression and a sustained inhibition of tumour growth. This antisense-inhibition of tumour growth was confirmed by the use of four distinct antisense constructs corresponding to the different N-terminal regions of RI_α codons. The growth inhibition may have been due to actions other than the blockage of RI_α expression, as nonspecific binding of the oligonucleotide or its degradation products to biological targets has been shown[29]. As discussed below, however, our data show that the antisense-inhibition of RI_α expression and modulation of protein kinase A isozymes are clearly related to the inhibition of tumour growth. Thus, at most, nonspecific binding of oligonucleotide probably played a minimal role in the observed growth inhibition.

In vivo pharmacokinetics studies in rodents showed a single intravenous dose of phosphorothioate oligodeoxynucleotide leaves the vascular space after 2–3 hours (phase α $t_{1/2}$ = 15–25 min), and its elimination from the body, which is almost completely urinary, requires 72 hours (phase β $t_{1/2}$ = 20–40 hours)[30]. Our results are in accordance with such pharmacokinetics of oligonucleotide and show a single s.c. dose of RI_α antisense produces an acute reduction in RI_α ($t_{1/2}$ RI_α = 31 hours (ref. 31)) content within 24 hours, and thereafter for 2–3 days. This reduction in RI_α triggered a compensatory increase in RII_β and an elimination of PKA-I activity.

The downregulation of PKA-I lasted for several days even after the RI_α suppression ceased, suggesting that RI_α may be functionally different, because it no longer formed the holoenzyme, PKA-I. We speculate this may be due to the following: (1) once RI_α is downregulated, the free C subunits complex with all of the available RII_α subunits to form PKA-II_α; (2) the remaining free C subunits trigger the synthesis of RII_β and form PKA-II_β; (3) RII has a greater half-life than RI (RII, $t_{1/2}$ = 125 hours; RI, $t_{1/2}$ = 31 hours (ref. 31)); therefore, once RII is synthesized, it remains in the cell for a longer time and favours complex formation with the C subunit as compared with RI; (4) the subsequently formed RI_α, after its initial suppression (due to the antisense), can no longer form PKA-I holoenzyme in the presence of the increased amount of PKA-II, which is favoured over PKA-I in its holoenzyme formation[5]; and (5) RI may be degraded and cannot form PKA-I. Although the exact mechanisms of action await future studies, our results showed that the antisense produced a biochemical imprint in tumour cells. The cells behaved like untransformed cells by making less PKA-I than PKA-II. This may be the basis for the suppression of tumour growth.

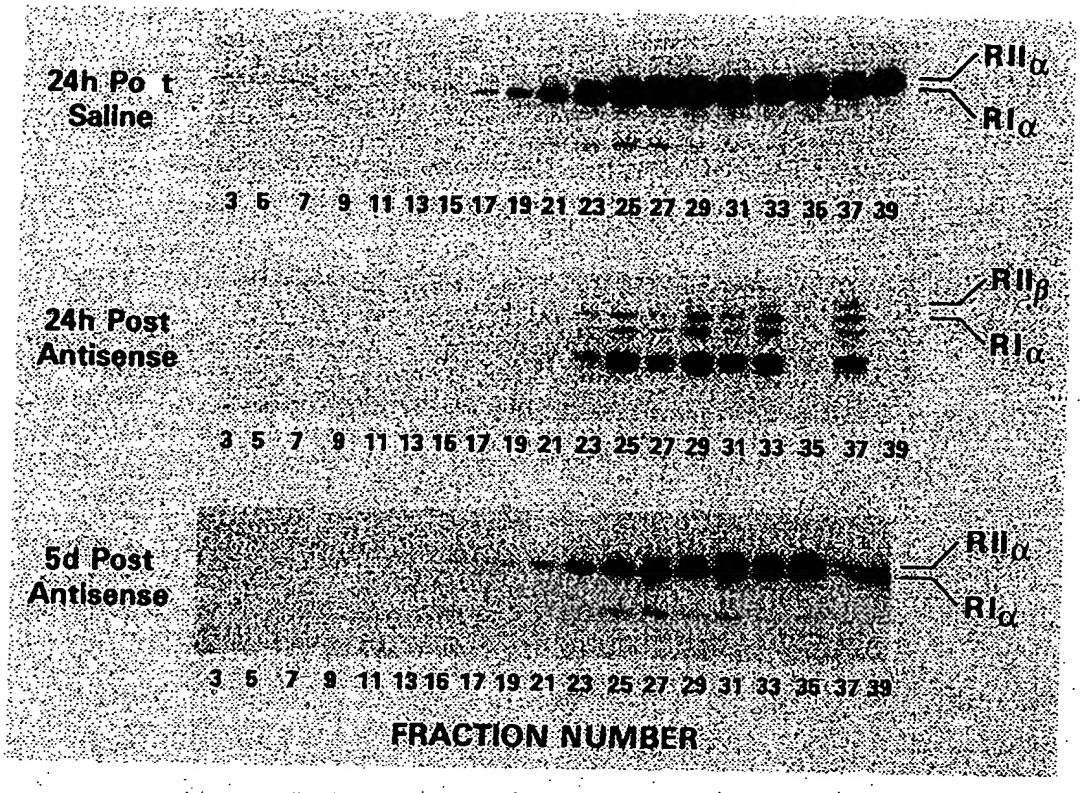

Fig. 4 PKA R subunit distribution in DEAE column fractions of tumours from the antisense- and saline-treated animals. The R subunit isoforms in DEAE column eluents (Fig. 3) were measured by photoaffinity labelling with 8-N_3-[^{32}P]cAMP and SDS–PAGE[20]. The data represent one of four separate experiments that gave similar results.

We have demonstrated that a single dose of RI_α antisense triggered the suppression of RI_α that preceded tumour growth inhibition. Importantly, the growth inhibition persisted, even after RI_α suppression ceased, as long as PKA-I (the RI_α-containing holoenzyme) downregulation was present. The single-injection antisense treatment introduced a programming in growth control in tumour cells, and thus was sufficient to produce a sustained inhibition of growth. This unexpected finding has a great impact on the application of antisense oligonucleotides as therapeutic agents, especially in terms of potency, targeting and cost. Our results suggest that an antisense like RI_α antisense, which is capable of producing a biochemical imprint for growth control in tumour cells, may require relatively infrequent repetitive dosing to maintain its inhibitory effect toward tumour growth *in vivo*.

Methods

Tumour growth and antisense treatment. LS-174T human colon carcinoma cells (1×10^6 cells) were inoculated s.c. into the left flank of athymic mice. The RI_α antisense phosphorothioate oligodeoxynucleotide (corresponding to the RI_α NH_2-terminus 8–13 codons (RI_α antisense) (5′-GCG-TGC-CTC-CTC-ACT-GGC-3′)) and control antisense (the same base composition as the RI_α antisense with the sequence jumbled) were kindly provided by T. Geiser (Lynx Therapeutics, Hayward, California). A single dose of RI_α antisense or control antisense (1 mg per 0.1 ml saline per mouse) or saline (0.1 ml per mouse) was injected s.c. into the right flank of mice when tumour size reached 80–100 mg, 1 week after cell inoculation. Tumour volumes were obtained from daily measurement of the longest and shortest diameters and calculation by the formula, $4/3\pi r^3$ where r = (length + width)/4. At each indicated time, two animals from the control and antisense-treated groups were killed, and tumours were removed, weighed, immediately frozen in liquid N, and kept frozen at –80 °C until used.

Photoaffinity labelling followed by immunoprecipitation of R subunits. The tumours were homogenized with a Teflon/glass homogenizer in ice-cold buffer 10 (Tris-HCl, pH 7.4, 20 mM; NaCl, 100 mM; NP-40, 1%; sodium deoxycholate, 0.5%; $MgCl_2$, 5 mM; pepstatin, 0.1 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.2 mM; aprotinin, 0.4 mg ml^{-1}; and soybean trypsin inhibitor, 0.5 mg ml^{-1}; filtered through a 0.45-μm pore size membrane), and centrifuged for 5 min in an Eppendorf microfuge at 4 °C. The supernatants were used as tumour extracts.

The amount of R subunits of PKA in tumours was determined by photoaffinity labelling with 8-N_3-[^{32}P]cAMP followed by immunoprecipitation with the R antibodies as previously described[20].

DEAE-cellulose column chromatography. Extracts (10 mg protein) of tumours from antisense-, control antisense- or saline-treated animals were loaded onto DEAE cellulose columns (1 × 10 cm) and fractionated with a linear salt gradient[22]. PKA activity was determined in the absence or presence of 5 μM cAMP[22]. cAMP-binding activity was measured by the method described previously and expressed as the specific binding[23].

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